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Neural retinas promote cell division and fibre differentiation in lens epithelial explants

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ABSTRACT

Histological analysis of lens epithelial cells from newborn rats co-cultured with neural retinas over a 12 day period showed stimulation of cell division and fibre differentiation. Cell division increased to a peak of 0.54 ± 0.13% at 6 days and then decreased to near zero at 12 days. Early signs of differentiation at 3 days included some cell elongation and weak fluorescence for β-crystallin. Differentiation was progressive and by 12 days there was further elongation and multilayering of cells with most cells fluorescing strongly for β-crystallin and some cells fluorescing strongly for γ-crystallin. Cell free, neural retina conditioned medium (NRCM) also stimulated epithelial cell division and fibre differentiation. The peak mitotic index was 0.12% at 2 days. Although there were no signs of differentiation at 3 days, by 12 days the majority of cells fluoresced strongly for β-crystallin and less than half fluoresced for γ-crystallin. Our interpretation of these results is that neural retinas release diffusable substances that stimulate division and differentiation of lens epithelial cells. The stronger proliferation and differentiation responses of epithelial cells in explants co-cultured with neural retinas is probably due to continual release of the factors and their build-up in the medium during the 12 day culture period.

INTRODUCTION

Two forms of cells comprise the vertebrate lens; fibre cells form the bulk of the lens and the anterior half of the lens is covered by a monolayer of epithelial cells. During life, epithelial cells proliferate and progeny of their divisions at the lens equator differentiate into fibre cells (1,2,3). There is a large body of evidence that shows that fibre cell differentiation is dependent on the influence of neural retina. In their classic experiment, Coulombre & Coulombre (4) turned lenses of chick embryos through 180° so that lens epithelium which normally faced the cornea now faced the retina. Under the influence of the new environment the epithelial cells elongated and gave rise to fibre cells. This was found in similar experiments with mice eyes and here it was also shown that growth of lens depended on the presence of neural retina (5). In vitro experiments with rat eyes showed that neural retinas grown on Millipore filters with lens epithelial cells stimulated β- and γ-crystallin synthesis (6) and other changes characteristic of fibre differentiation in rats. Beebe et al. (7) reported that a factor in the vitreous, lentropin, promotes lens fibre cell differentiation in chick eyes. One interpretation of these experiments is that a factor in the vitreous that arises from neural retina stimulates equatorial epithelial cells to differentiate into fibres.

Arruti and Courtois (8) reported that an extract of bovine neural retina stimulated cell division in cultured lens epithelial cells. Here we investigate the influence of neural retina and neural retina conditioned medium (NRCM) from rats on both cell division and crystallin synthesis in cultured rat lens epithelia. This work extends previous findings (6) and provides evidence that neural retina produces a diffusable factor or factors that can sti-

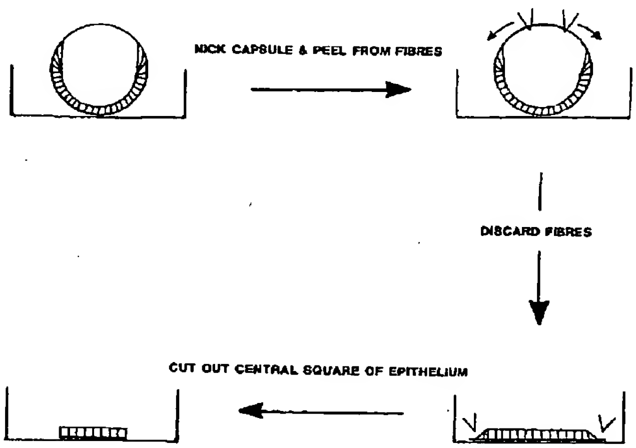

Figure 1 Procedure for explanting lens epithelial cells. The lens epithelial cells are cultured on their capsule which is pinned on bottom of petri dish.

mulate epithelial cells to divide as well as undergo changes characteristic of fibre cell differentiation.

MATERIALS AND METHODS

Eyes were removed from newborn (1-3 day) rats and placed in medium 199 (Flow Laboratories) containing streptomycin sulphate and benzylpenicillin sodium. Neural retinas and lenses were dissected out under a dissecting microscope in a laminar flow cabinet. Watchmakers forceps were used to tear away the fibrous tunic and separate lens from retina. Lenses were gently rolled on sterilized filter paper to remove any cells adhering to the lens capsule. Lens epithelial cells were then carefully separated from lens fibre cells as shown in Figure 1. In the final step of the explantation procedure the tissue pinned to the bottom of the culture dish was trimmed so that only a square of central epithelial cells remained. This ensured that incipient fibre cells from the lens equator were excluded from the explant. Explants were cultured in pairs in 30 mm plastic disposable culture dishes (Lux) containing 2 mls medium in an incubator at 37°C in 5% CO_2/air mixture (Forma Scientific).

Some explants were grown along with 4 neural retinas/dish. Others were grown in neural retina conditioned medium (NRCM). This was prepared by culturing 2 neural retinas/ml of medium 199 for 3 days. The medium was then centrifuged at 200 g for 10 mins to spin down retina cells. The supernatant was then filtered through a 0.2 µm Amicon 'sterilet' to sterilize the medium and to remove any remaining cells.

Histology and immunofluorescence

Explants were fixed in Carnoys (1:99, acetic acid/alcohol) for 5 minutes, washed in absolute alcohol and hydrated. Explants were loosened from the bottom of the culture dish with a scalpel, the water was poured off and a drop of 3% molten agar placed on top of the explants. When the agar had hardened it was removed, along with adhering explants, from the bottom of the dish. The agar block was dehydrated and embedded in paraffin wax. Sections were cut at 5 µm and anti-crystallin antibodies were used to localise α-, ß- and γ-crystallins as previously described (9). Serial sections were used to compare distribution of α-, ß-, and γ-crystallins in one explant. The numbers of cells fluorescing after treatment by each of the 3 antibodies were counted in sections from 3 different regions of each explant. The total numbers of cells in the sections were estimated from counts in an adjacent section that had been stained with haematoxylin and phloxine. Cell counts were made largely by counting nuclei. However, in the explants that had differentiated, many of the large elongated or globular cells had no distinct nuclei and the borders were often indistinct. For this

reason the cell counts were approximate; therefore, as described in an earlier study (6), percentages of fluorescing cells were placed in five classes: 1-5%, 6-25%, 26-50%, 51-75%, 76-100%.

Mitotic indices

Explants were cultured for 2,4,6,9 and 12 days then fixed in Carnoys for 5 minutes, hydrated and stained with haematoxylin and phloxine. The explants were mounted directly in phosphate buffered saline in 10% glycerol. The rim of the culture dish was removed with scissors to facilitate viewing under the microscope. The mitotic index was calculated for each explant by counting the proportion of mitotic cells in a 0.34 mm² area using an eye piece graticule. The explant was moved so that the graticule covered nine different areas of the explant. The first measurement was taken in the centre of the explant, then 2 areas extending outwards from each of the four sides of the central area were counted.

The mitotic index was expressed as percentage of dividing cells. All statistical analyses of the results were carried out according to methods in Snedecor and Cochran (10).

RESULTS

Influence of neural retinas on cell division

Lens epithelial explants grown in control medium 199 show a decrease in mitotic activity from 0.09 ± 0.05% at the time of setting up the explant to 0.02 ± 0.01% at 3 days of culture (Fig. 2). From this time onwards cell division is only occasionally detected in explants. In contrast explants grown in combination with neural retinas increase to 0.22 ± 0.16% mitotic activity by 4 days and reach a peak of 0.54 ± 0.13% at 6 days. From 6 days onwards mitotic activity diminishes in these cultures to 0.03 ± 0.04% at 12 days of culture.

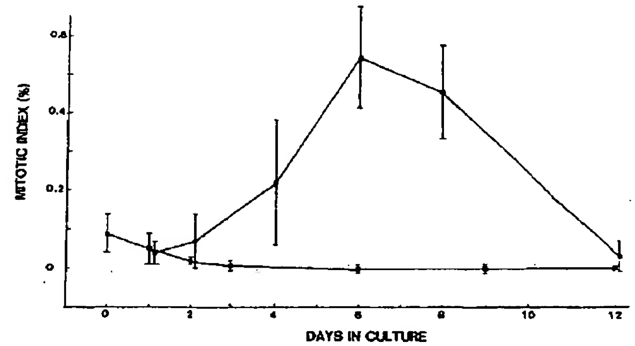

Figure 2 Influence of neural retinas on epithelial cell division over 12 days of culture (-■- controls, -●- explants + neural retinas).

Influence of neural retinas on fibre differentiation

Epithelial explants grown in control medium 199 for 3 days retained an epithelial morphology as judged by their squamous cuboidal shape and indented, condensed nuclei (Fig. 3). Localisation of crystallins by immunofluorescence showed that 76-100% cells strongly fluoresced for α-crystallin and there was no fluorescence for ß- and γ-crystallins (Table 1). Therefore control explants had a morphology and crystallin composition characteristic of lens epithelial cells in vivo. (9)

All explants grown in combination with neural retinas for 3 days were columnar in shape and 76-100% cells strongly fluoresced for α-crystallin (Fig. 3, Table 1). In 3 out of 4 explants 76-100% cells fluoresced for ß-crystallin. Fluorescence was variable in intensity with some cells fluorescing strongly and others weakly. In the remaining explant no cells fluoresced significantly above background to be judged positive for ß-crystallin. In 3 out of 4 explants there was no fluorescence for γ-crystallin but in one explant

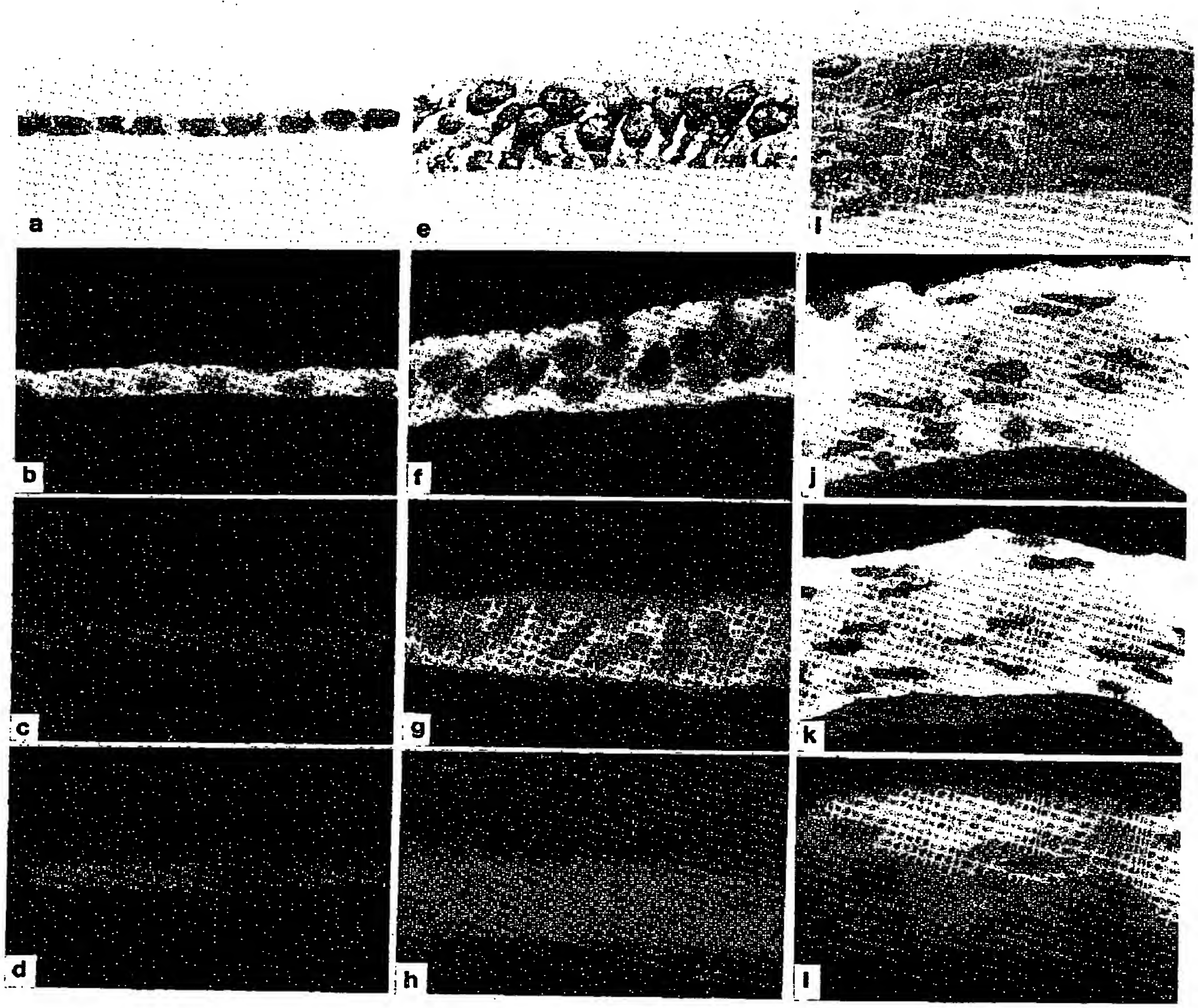

Figure 3 Typical immunofluorescent localisation of crystallins in explants grown in medium 199 for 3 days (a-d) and in combination with neural retinas for 3 days (e-h) and 12 days (i-l). Serial sections were stained with haematoxylin and phloxine (a,e, and i) and immunofluorescence was used to localise α-crystallin (b,f and j), β-crystallin (c,g, and k) and γ-crystallin (d,h, and l). After 3 days with neural retinas the cells elongated slightly, and showed weak but significant fluorescence for β- and no significant fluorescence for γ-crystallin. After 12 days with neural retinas the cells had elongated substantially and formed multilayers. They all fluoresced strongly for β- and some fluoresced strongly for γ-crystallin. X 720.

1-5% cells weakly fluoresced for γ. After 12 days incubation with neural retinas the cells had elongated, many of them running parallel to the capsule, and formed multilayers. In all explants 76-100% of cells fluoresced for α-, β- and

TABLE 1

crystallin composition of explants incubated with neural retinas and NRCM.

Treatment	Incubation time (days)	Crystallins α	β	γ
Medium 199 (controls)	3	+++++*(4)*	0 (4)	0 (4)
NRCM	3	+++++(4)	0 (4)	0 (4)
Medium 199+n.retinas	3	+++++(4)	+++++(3) 0 (1)	+ (1) 0 (3)
Medium 199 (controls)	12	+++++(4)	+ (1) 0 (3)	0 (4)
NRCM	12	+++++(4)	+++++ (4)	+++ (4)
Medium 199+n.retinas	12	+++++(4)	+++++ (4)	+++++ (4)

*+1-5%, ++6-25%, +++26-50%, ++++51-75%, +++++76-100%

* numbers in parenthesis indicate number of explants

γ-crystallins. Fluorescence for α- and ß-crystallins was strong in all cells but fluorescence for γ was variable with the strongest fluorescence being present in the more elongated cells usually located at the edges of the explants.

Thus neural retinas stimulated the synthesis of ß- and γ-crystallins. Although there was some variation between explants in the timing of ß- and γ-crystallin synthesis, ß-crystallin was consistently detected earlier than γ-crystallin.

Influence of NRCM on cell division

To investigate if the stimulation of cell division in lens epithelial explants was mediated by a diffusable substance from neural retina, explants were grown in NRCM. Explants were set up and left for 3 days in medium 199 to allow mitotic activity to drop to near zero (see Fig. 4) before NRCM was added. Exposure of explants to NRCM showed that cell division was stimulated in some, but not all explants. Moreover there was a wide range of mitotic indices in those explants that were stimulated. For example, after 4 days exposure to NRCM explants showed mitotic activity ranging from 0.01% to 0.38%. Nevertheless since controls mostly showed no mitotic activity the means of mitotic indices in explants grown for 2,4,6 and 9 days were greater than controls. The difference between mitotic indices of controls and explants grown in NRCM for 2 days or 4 days was highly significant (P=0.01, Mann-Witney rank test).

It is interesting that some explants did not proliferate and others showed a wide range of proliferative responses when treated with the same NRCM. It was noted that cell density also varied between explants. For those explants that showed mitotic activity there was a significant negative correlation between cell density and mitotic index ($P<0.01$), i.e. highest mitotic indices were in explants with lowest cell densities. Moreover, we recently found that by modifying the explantation procedure to include a 10 min incubation of lenses at 5%, CO_2 prior to epithelial explantation, explants had significantly higher cell concentrations

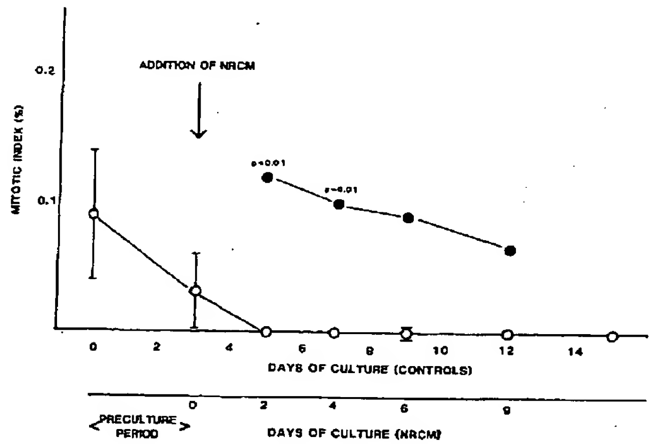

Figure 4 Influence of NRCM on epithelial cell division over 12 days of culture. (-o- controls, -●- explants + NRCM). Epithelial explants were cultured for 3 days before introducing NRCM.

-3529 ± 206 cells/mm² compared with 2882 ± 294 cells/mm² using the standard explantation procedure. In one experiment using the modified procedure, out of twenty explants grown with NRCM for 2 and 4 days only 2 explants showed any mitoses. Both explants had a mitotic index of 0.01%. These observations that lower levels of cell division are induced in more crowded cultures are consistent with reports that cell density influences cell growth. For example, Dulbecco (11) showed that when a strip of cells is removed from a monolayer untransformed culture the remaining cells, particularly those around the wound, are stimulated to divide by serum factors.

In the case of the lens epithelium the greater mitotic response in explants co-cultured with neural retinas is probably due to a stronger proliferation influence which is dependent on the continuous presence of neural retina cells.

Influence of NRCM on fibre differentiation

Explants grown in NRCM for 3 days retained a cuboidal morphology but by 12 days had elongated and formed multilayers as did explants grown in combination with neural retinas (see Fig. 3). Localisation of crystallins by immunofluorescence at 3 days of culture showed that in all explants 76-100% cells fluoresced strongly for α but no cells fluoresced for β- and γ-crystallins. By 12 days in all explants 76-100% cells fluoresced strongly for β and 26-50% cells fluoresced for γ-crystallin. The fluorescence for γ was variable with some cells fluorescing strongly and others weakly. As with explants grown with neural retina the strongest fluorescing cells were predominantly located at the edges of the explants.

DISCUSSION

Neural retinas stimulate both cell division and fibre cell differentiation in lens epithelial explants. Over a 12 day culture period the average mitotic index of explants grown with neural retinas is stimulated to a peak of 0.54 ± 0.13% at 6 days. After 3 days with neural retinas, β-crystallin is detected in most cases in 76-100% cells but γ-crystallin is only occasionally detected at this time. At equivalent times in controls there is al-

most no cell division and no cells contain β- or γ-crystallins. The proportion of cells containing β-crystallin in explants grown with neural retinas is about the same at 3 days as at 12 days, but the overall fluorescence, and therefore content of β-crystallin, is much greater due to greater intensity of fluorescence in cells and greater size of explants. The proportion of cells containing γ-crystallin in these explants increases substantially from 3 to 12 days. These results show that crystallin synthesis and accumulation to detectable levels is sequential, with β-crystallin appearing before γ-crystallin.

It is interesting that the proportion of cells fluorescing for β-crystallin does not change significantly after 3 days but that the number of cell divisions continues to increase up to 6 days. It is possible that some of the increasing number of dividing cells up to 6 days may synthesise β-crystallins. Alternatively, cell division and β-crystallin synthesis may be mutually exclusive and cell division may be restricted to a small population of cells that retain epithelial characteristics but are limited in their replicative potential after 6 days, either by a decrease in the abundance of the proliferation factor or by their response to the factor. It is not possible to clearly distinguish between these possibilities at this stage in the work.

NRCM stimulates both cell division and fibre cell differentiation in lens epithelial explants. Stimulation of cell division is highest after 2 days exposure to NRCM and diminishes from 2 days onwards. This, plus the observation that the peak proliferative response induced by NRCM (0.12% mitoses) is substantially lower than the peak response induced by neural retinas (0.54% mitoses) indicates that the potency of the proliferation influence depends on the presence of neural retinas and continuous modification of the medium during explant culture.

The stimulation of β- and γ-crystallin synthesis by NRCM is also weaker than that provided by neural retinas as evidenced by the later appearance of β-crystallin, and by detection of γ-crystallin in fewer cells at 12 days. This also indicates that the potency of the differentiation influence depends on the presence of neural retinas and continuous modification of the medium during explant culture.

Our interpretation of these results is that neural retinas release a diffusable factor, or factors, that stimulates lens epithelial cells to divide and differentiate into fibres. It is not clear whether we are dealing with separate entities or if the same factor, or factors, brings about two different responses. Neither is it known how our rat neural retina factors are related to a bovine growth factor derived from different eye tissues (12,13). This factor as well as stimulating cell division also brings about changes in cell shape (14). However, since there has been no analysis of crystallin content in these cells it is not clear how these shape changes are related to fibre differentiation.

Investigation of the relationship between proliferation and differentiation forms part of a study on factors controlling these events in rat lens epithelial explants.

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